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*Full Length Research Paper*

# Microbial conversion of Cr (VI) in to Cr (III) in industrial effluent

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**Bacterial strains (CrT-11, CrT-12, *Bravibacterium* sp. CrT-13, CrT-14) were isolated from the effluents of tanneries. All strains could resist very high concentration of $K_2CrO_4$ that is up to 40 mg $ml^{-1}$ on nutrient agar and 25 mg $ml^{-1}$ in nutrient broth. They have wide pH (5 to 9) and temperature (24 to 42˚C) growth range. They exhibited multiple metals (Ni, Zn, Mn, Cu, Co, Pb) and antibiotics (streptomycin, ampicillin, tetracycline, kanamycin, chloramphenicol) resistances. All the strains were able to reduce Cr (VI) in to Cr (III) aerobically. *Bravibacterium* sp. CrT-13 accumulates and reduce more Cr (VI) at all the concentrations applied in comparison to the other strains. These bacterial strains also take up and reduce Cr (VI) present in industrial effluents, and their reduction potential was not significantly affected in the presence of different metallic salts**.

**Key words:** Cr (VI) reduction, bacteria, industrial effluent, heavy metals.

## INTRODUCTION

The current pattern of industrial activity alters the natural flow of materials and introduces novel chemicals into the environment. In areas polluted as a result of industrial activity, concentrations of toxic substances often exceed the levels normally found in soil, waterways, and sediment. When toxic substances accumulate in the environment and in food chains, they can profoundly disrupt biological processes.

Hexavalent chromium, Cr (VI), is the toxic form of chromium released during many industrial processes including electroplating, leather tanning, and pigment manufacture. Chromium is an essential element required for normal carbohydrate and lipid metabolism (Anderson, 1998). Its deficiency leads to increase in risk factors associated with diabetes and cardiovascular diseases including elevated circulating insulin, glucose, triglycerides, total cholesterol and impaired immune function. Contrary to deficiency symptoms, several factors make chromate contamination as a matter of intense concern, particularly its toxic, mutagenic (Cheng and Dixon, 1998), carcinogenic (Shumilla et al., 1999) and terartogenic (Asmatullah et al., 1998) effects. Removal of this toxic form of chromium by its reduction to the much less harmful and physically immobile state Cr (III) is now our concern.

Bioremediation of soluble hexavalent chromium can be obtained by utilizing microbes in wastewaters. Several bacterial strains (*Pseudomonas ambigua*, *Desulfovibrio vulgaris*, *Enterobacter cloacae* HO-1, *Alcaligenes eutrophus* , *Dinococcus radiodurans* R1) has been described for their ability to reduce hexavalent chromium into insoluble low valence form Cr (III) both aerobically and aerobically (DeLeo and Ehrlich, 1994; Fredrickson et al., 2000; McLean and Beveridge, 2001). Hence the

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chromate reducing bacteria are most crucial for the immobility of soluble hexavalent chromium in the environment. The goal of this study was to develop a system to describe chromate reduction alone and in the presence of other toxic contaminants.

## MATERIALS AND METHODS

### Microorganisms Characterization

Selected strains were characterized morphologically, biochemically, and physiologically following Gerhardt et al. (1994). The impact of potassium chromate, pH and temperature on the growth of these strains was observed in nutrients broth. Fresh inoculum from overnight cultures adjusted to $10^8$ cells $ml^{-1}$ was added to nutrient broth containing different concentrations of potassium chromate. Cultures were grown at 37 ºC for 24 h and growth was monitored at 600 nm. Different heavy metals ($NiSO_4$, 500 µg $ml^{-1}$; $ZnSO_4$, 700 µg $ml^{-1}$; $MnSO_4$, 1500 µg $ml^{-1}$; $CuSO_4$, 1000 µg $ml^{-1}$; $CoCl_2$, 500 µg $ml^{-1}$; $HgCl_2$, 50 µg $ml^{-1}$ and $Pb(NO_3)_2$, 1000 µg $ml^{-1}$), and antibiotics (streptomycin, 500 µg $ml^{-1}$; ampicillin, 300 µg $ml^{-1}$; tetracycline, 30 µg $ml^{-1}$; kanamycin, 40 µg $ml^{-1}$; chloramphenicol, 5 µg $ml^{-1}$) were used to check the multiple metals and antibiotics resistance profile of these strains.

### 16S rRNA gene sequencing

To confirm taxonomic identity, one strain (CrT-13) was selected for 16S rRNA gene sequencing. DNA was extracted and a part (500 bp) of the 16S rRNA gene was amplified, and sequenced using fluorescent di-deoxy terminator cycle sequencing chemistry in an ABI PRISM® (automated DNA sequencer). The data was compared to the MicroSeq® databases (ACCUGENIX™ Newark DE 19702).

### Cr (VI) uptake

For uptake experiment three initial $K_2CrO_4$ concentrations, 100, 500, and 1000 µg $ml^{-1}$, were used. The inoculum was prepared as follow.
i) One gram of fresh cell pellet was dried at 60ºC for 48 h (dried cells). ii) Same amount of pellet was heat-killed at 121ºC for 20 min (Heat killed). iii) Same amount was used untreated (live cell mass).

All experiments were conducted in 250 ml conical flasks containing 100 ml of chromium solution. Before mixing the bacterial strains, the pH of the chromium solution was adjusted to the required value with 0.01N NaOH. Bacterial cell suspension was added to metal solution and was placed in an incubating shaker at 150 rpm. At regular time intervals, samples were taken aseptically and were centrifuged at 10,000 rpm for 10 min at 4ºC. Pellet obtained were digested and the amount of chromium accumulated was determined spectrophotometrically at 540 nm in a spectrophotometer using diphenylcarbazide as the complexing agent (APHA, 1990).

### Cr (VI) reduction

To check the chromate reduction potential of these isolates, Deleo and Ehrlich (1994) medium (Grams per liter: Tryptone 10, Yeast extract 5, NaCl 5, Citric acid 1, $NaH_2PO_4$ 6.9) was used. Three initial chromate concentrations, 100, 500, and 1000 µg $ml^{-1}$ and two cells concentrations, 2.4 X $10^7$ and 9.6 X $10^7$ cells $ml^{-1}$ were used. Cultures were inoculated from cell growing in log phase. Reduction experiments were conducted in 250 ml conical flasks containing 100 ml of medium. Cultures were placed in an incubating shaker at desired temperature. The initial pH of these experiments was 7. At selected points, samples were withdrawn aseptically and were processed to check the amount of hexavalent chromium reduced. Reduction of chromate was measured by colorimetric method following DeLeo and Ehrlich (1994) using spectrophotometer (Model S-300 DL, R & M marketing, England) at 540 nm. Each treatment was conducted in triplicate.

### Effect of Heavy Metals on Cr (VI) Reduction

The isolates were also assessed for their ability to reduce Cr (VI) in the presence of different heavy metals (Zn, 200 µg $ml^{-1}$; Ni, 200 µg $ml^{-1}$; Mn, 200 µg $ml^{-1}$; Cu, 200 µg $ml^{-1}$; Co, 50 µg $ml^{-1}$ and Ag, 50 µg $ml^{-1}$). The amount of Cr (VI) reduced was measured as described above. Each treatment was conducted in triplicate.

### Cr (VI) Reduction in industrial effluent

In order to check the hexavalent chromium reduction efficiency of these strains in industrial effluents, samples were collected in sterilized bottles. The physico-chemical parameters of the effluent sample was as follow: pH 5-6, temperature 27-29ºC, Cr (VI) 300 µg $ml^{-1}$; Fe 21 µg $ml^{-1}$; Cu 10 µg $ml^{-1}$; Zn 4 µg $ml^{-1}$; Ni 11 µg $ml^{-1}$; Co 2 µg $ml^{-1}$; Pb 1 µg $ml^{-1}$; Mn 1 µg $ml^{-1}$. In this effluent sample some nutritional requirements for bacterial growth were also added. Two different dilution of effluent sample were used; sample I and sample II containing 150 and 300 µg $ml^{-1}$ of Cr (VI), respectively. After inoculation these samples were incubated for 40 h at 37ºC. Cr (VI) reduced was measured as describe above.

## RESULTS

### Strains isolation and characterization

The current study deals with four bacterial strains (CrT-11, CrT-12, *Bravibacterium* sp. CrT-13 and CrT-14). All were isolated from the effluents of tanneries. Strains CrT-11, CrT-12 and CrT-14 were gram-negative motile rods while strain *Bravibacterium* sp. CrT-13 was gram positive. All were aerobic in nature. They were unable to hydrolyse starch and arginine. Except strain *Bravibacterium* sp. CrT-13, all were able to reduce nitrate into nitrite. None of them could denitrify (Table 1).

Nevertheless, the growth of all the strains decreased with increasing concentration of chromate salt, but all of them could grow in the presence of 25 mg $ml^{-1}$ of $K_2CrO_4$. *Bravibacterium* sp. CrT-13 exhibited relatively poor growth at all the concentrations. In the presence of chromate (1 mg $ml^{-1}$) the lag phase in the strains CrT-11, CrT- 12 and *Bravibacterium* sp. CrT- 13 prolonged (Figure 1b). The temperature preference remains the same (37ºC), both in the presence and absence of chromate salt. Growth of the strains was much reduced at low temperature (24ºC; Figure 2a). These strains preferred pH 7 both in simple nutrient broth and in chromate supplemented media. In the presence of chromate, the growth was very poor at pH 5 as compared to chromate

**Table 1.** Morphological and biochemical characteristics of bacterial isolates.

| Characteristics | Strains | | | | Characteristics | Strains | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CrT-11 | CrT-12 | CrT-13 | CrT-14 | | CrT-11 | CrT-12 | CrT-13 | CrT-14 |
| Colony shape | Circular | Circular | Circular | Circular | Voges Proskauer | - | - | - | - |
| Colony elevation | Convex | Convex | Convex | Convex | Nitrate reduction | + | + | - | + |
| Colony size (mm) | 2.4 | 1.9 | 1.3 | 1.4 | Denitrification | - | - | - | - |
| Colony margin | Entire | Rods | Rods | Rods | $H_2S$ production | - | - | - | - |
| Cell shape | Rods | L.Yell | Yellow | L.Yell | OF | Aerobic | Aerobic | Aerobic | Aerobic |
| Cell size | 1.0-0.4 | 1.2-0.5 | 1.2-0.6 | 2.0-0.4 | Acid from sucrose | - | - | - | - |
| Gram staining | -ve | -ve | +ve | -ve | Acid from maltose | - | - | - | - |
| Capsules staining | - | - | - | - | Acid from mannitol | - | - | - | - |
| Spore staining | - | - | - | - | Acid from lactose | - | - | - | - |
| Urease test | - | - | - | - | Growth on malonate | - | - | - | - |
| Motility | + | + | + | + | Growth on EMB | + | + | - | + |
| Catalase | + | + | + | + | MacConkey agar | + | + | + | + |
| Cytochrome | + | + | - | + | Brillient green bile | - | - | - | - |
| Arginine hydrolysis | - | - | - | - | King'A | - | - | - | - |
| Starch hydrolysis | - | - | - | - | King'B | - | - | - | - |
| Methyl red | - | - | - | - | Plasmid | + | + | + | + |

**Table 2.** Heavy metals resistance profile of chromium resistant bacterial isolates.

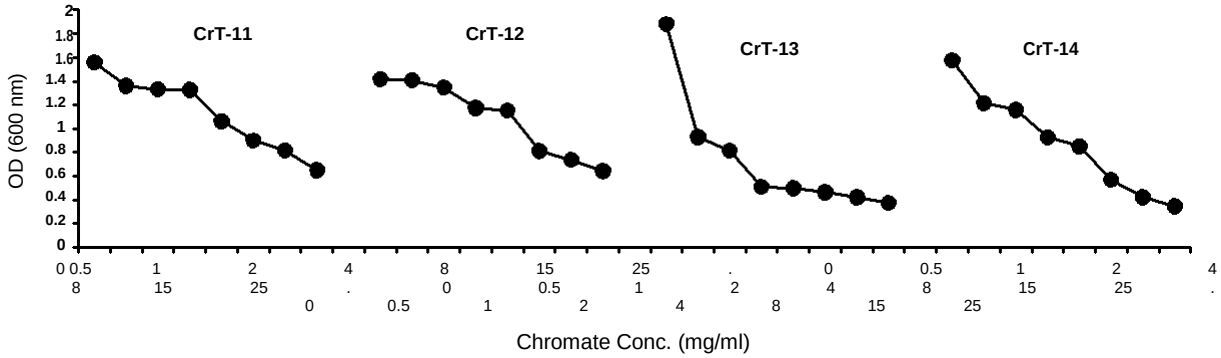
| Strains | Heavy metals (µg ml $^{1}$) | | | | | |
|---|---|---|---|---|---|---|
| | $NiSO_4$ | $ZnSO_4$ | $Pb(NO_3)_2$ | $CuSO_4$ | $CoCl_2$ | $MnSO_4$ |
| **CrT-11** | 500 | 500 | 1000 | 1000 | 300 | 1500 |
| **CrT-12** | 700 | 700 | 1000 | 1000 | 300 | 1500 |
| **CrT-13** | 400 | 500 | 1000 | 1000 | 200 | 1500 |
| **CrT-14** | 400 | 400 | 1000 | 1000 | 300 | 1500 |

**Table 3**. **A.** Effects of different heavy metals on the reduction potential of the strains. **B.** Chromate reduction in industrial effluents. a*. Initial chromate concentration 150 µg ml $^{1}$. b**. Initial chromate concentration 300 µg ml $^{1}$.

| Source | Strains | | | |
|---|---|---|---|---|
| | CrT-11 | CrT-12 | CrT-13 | CrT-14 |
| **A. Metals** (µg ml $^{1}$) | **% Cr (VI) Reduced** | | | |
| Control | 28.24% | 36.20% | 24.50% | 27.20% |
| Ni (200) | 28.00% | 38.60% | 24.60% | 28.50% |
| Mn (200) | 30.20% | 34.20% | 25.60% | 29.40% |
| Zn (200) | 31.40% | 37.00% | 27.50% | 31.40% |
| Cu (200) | 28.21% | 36.50% | 25.10% | 23.41% |
| Co (50) | 26.20% | 34.12% | 24.20% | 24.96% |
| **B. Industrial effluents** | | | | |
| a* | 71.10% | 93.12% | 68.20% | 79.00% |
| b** | 56.03% | 72.12% | 47.22% | 58.66% |

free nutrient broth (Figure 2b). Besides chromate, they also showed multiple metals and antibiotics resistance (Table 3). Nevertheless tolerance profile of different strains was different. All bacterial strains were sensitive to $HgCl_2$ at 50 µg ml $^{1}$ but their tolerance towards others metals was very high (Table 2). These strains show sensitivity to 500 µg ml $^{1}$ of streptomycin and 40 µg ml $^{1}$ of kanamycin. All the strains are resistant to 25 µg ml $^{1}$ of tetracycline. Excluding *Bravibacterium* sp. CrT-13, these strains are also resistant to 300 µg ml $^{1}$ of ampicillin.

**a.**




**b**.

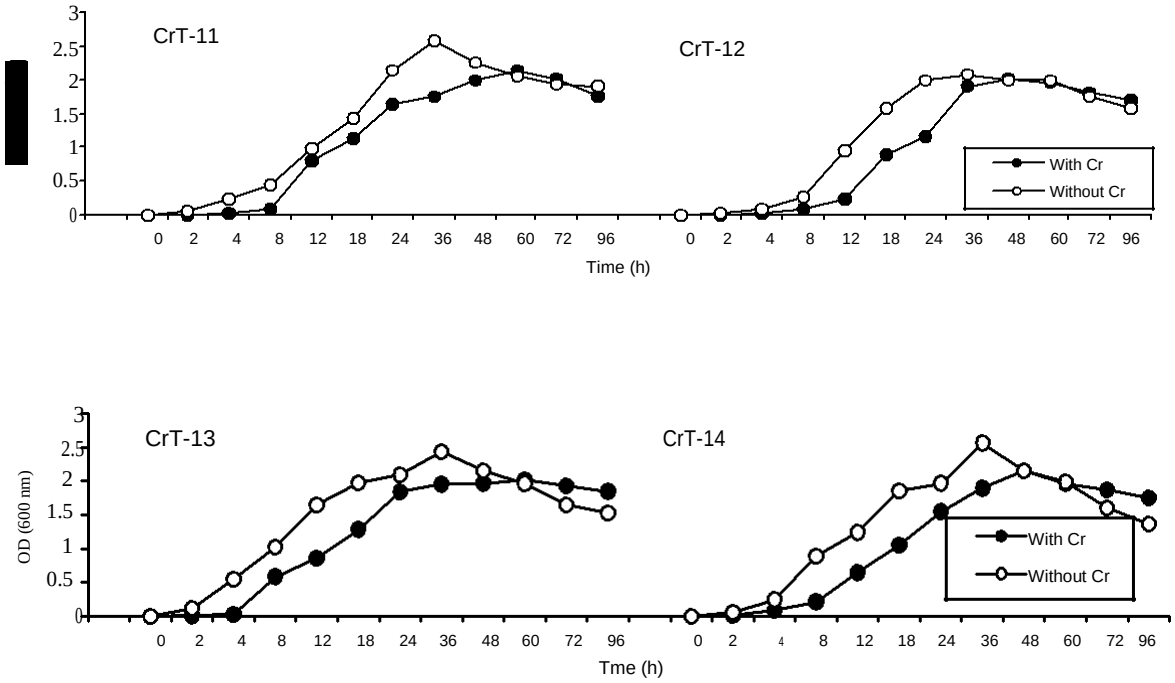



**Figure 1.** Growth responses of bacterial strains a) at different concentration of potasssium chromate, b) after different time of incubation in the presence and absence of chromate (1 mg ml $^{1}$).

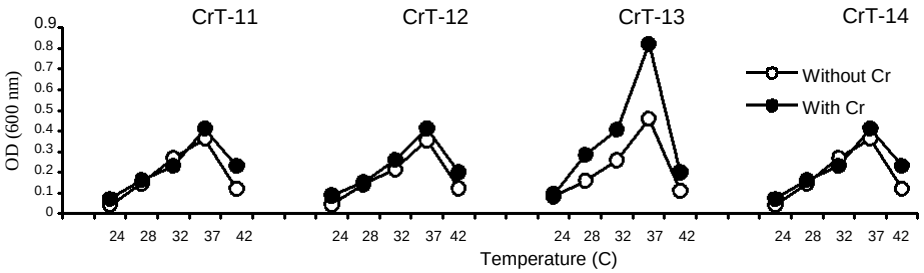
## Strain identification through 16S rRNA gene sequencing

The 16S rRNA gene is the most widely accepted gene used for bacterial classification and identification. Signature nucleotides of 16S rRNA genes allow classification even if a particular sequence has no match in the database, since otherwise unrecognizable isolates can be assigned to phylogenetic branches at the class, family, genus, or subgenus levels. The results of 16S rRNA gene sequencing are presented in the Figure 5. Identification of the isolate based on its partial (500 bp) 16S rRNA gene sequences assigned it to the gram-positive, *Bravibacterium* sp. CrT-13.

## Chromium uptake

The ability of cells to accumulate toxic hexavalent chromium from solution was determined at different (100, 500 and 1000 µg ml $^{1}$) chromate concentrations. Living cells showed higher chromate uptake than heat killed and dried cell mass. In the first fifteen minutes of contact time, the amount of chromium accumulated by the living cells of strain CrT-11 was 2.8, 3.7 and 13.9 mg g$^{-1}$ dry weights at an initial chromate concentration of 100, 500 and 1000 µg ml$^{-1}$, respectively. In heat-killed cells, the accumulations were 3.3, 3.6 and 12.6 mg g$^{-1}$ dry weights. The amount of chromium accumulated by the dried cell mass is 2.2, 3.1 and 8.3 mg g$^{-1}$ dry weight. It was

**a.**




**b.**

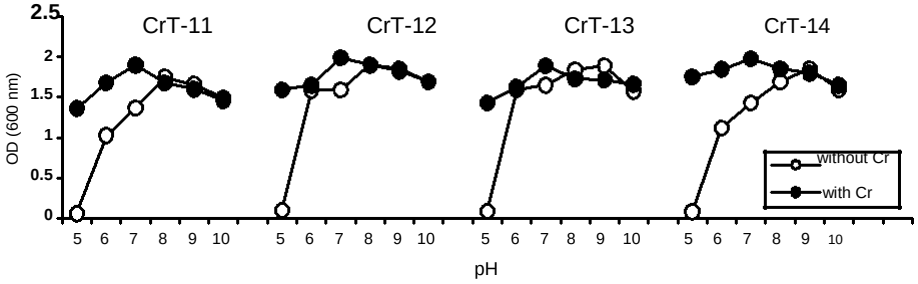



**Figure 2.** Growth responses of bacterial strains in chromate supplemented (1 mg ml $^{1}$) and chromate free nutrient broth at different (a) temperatures (24-42°c) and (b) growth pHs (5-9).

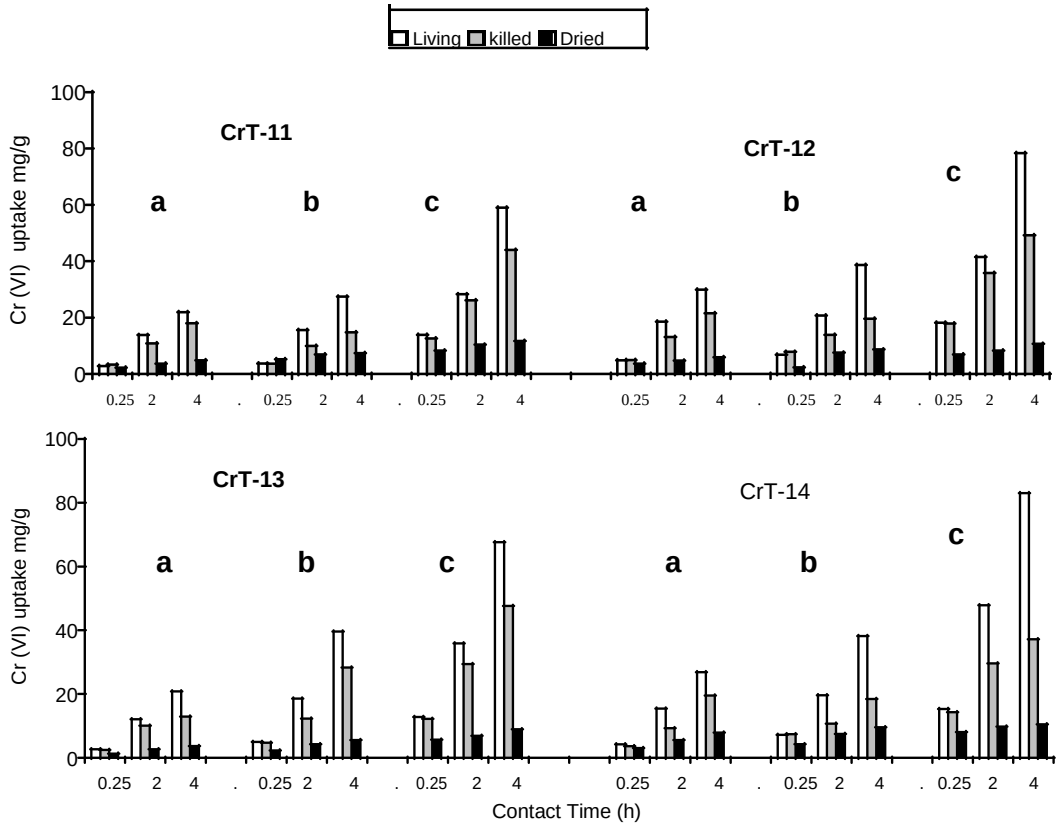



**Figure 3.** Uptake of potassium chromate at three initial $K_2CrO_4$ concentrations (a) 100, (b) 500 and (c) 1000 µg ml $^{1}$). Cells were used as dried, heat killed and as live.

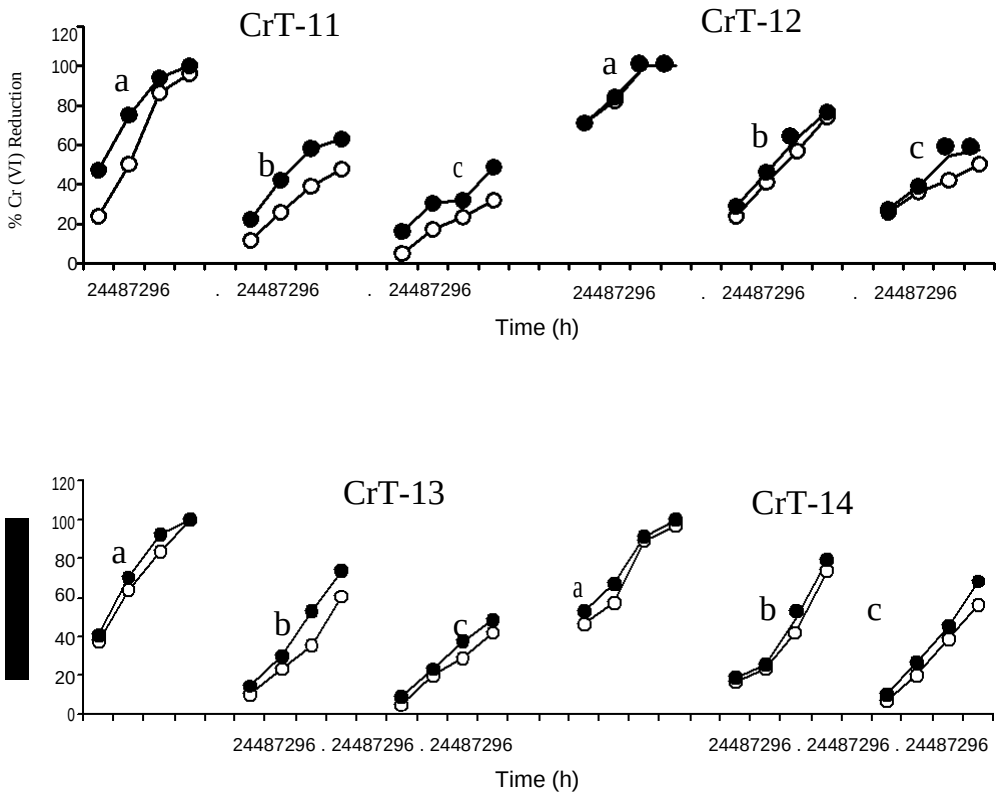



**Figure 4.** Reduction of $K_2CrO_4$ by Bacterial strains at three level of chromate. Reduction was monitor after 24, 48, 72 and 96 h of growth incubation. Open circle (o) represent 2.4 X $10^7$ cells $ml^{-1}$ and solid circle (●) represent 9.6 X $10^7$ cells $ml^{-1}$ used for initial inoculation. (a) Cr (VI) 100 µg $ml^{1}$; (b) Cr (VI) 100 µg $ml^{1}$; (c) Cr (VI) 1000 µg $ml^{1}$.

observed that after 2 and 4 h, chromium accumulated by the living cells was much more higher, specially at higher concentration relative to the other two categories i.e., heat-killed and dried cells. In this strain (CrT-11), after 4 h contact time the uptake values of 21.9, 27.4 and 59.0 mg $g^{-1}$ dry weight were observed in the living cells when initial metal solution was 100, 500 and 1000 µg $ml^{1}$, respectively, but in case of dried cell mass the values were 4.8, 7.4 and 11.7 mg $g^{-1}$ dry weight which are much less compared with living cell mass. Almost same trend was observed in others three strains in this respect. In all cell types, the amount of chromium accumulation increased as contact time of cells with metal solution increased.

## Cr (VI) reduction

Medium containing three levels of initial $K_2CrO_4$ concentrations, 100, 500 and 1000 µg $ml^{1}$ was inoculated with two different inoculum size, 2.4 X $10^7$ and 9.6 X $10^7$ cells $ml^{-1}$. The reduction of hexavalent chromium by these bacterial strains in the medium resulted in the production of offwhite residues that was the sign of chromate reduction. After 72 h samples containing initial Cr (VI) concentration of 100 µg $ml^{1}$ with inoculum of $2.4^7$ and $9.6^7$ cells $ml^{-1}$, CrT-11 reduces almost 74.12 and 86.25% of Cr (VI), respectively, while strain CrT-12 completely reduce all the chromate at the same contact time. The reduction pattern of all the strains are depicted in Figure 4.

## Cr (VI) reduction in industrial effluent

Cr (VI) reduction potential of these strains was also checked directly in the industrial effluent sample collected from a highly polluted metal finishing setup. In a sample that contains an initial chromate concentration of 150 µg $ml^{1}$ along with other pollutants, CrT-11, CrT-12, *Bravibacterium* sp. CrT-13 and CrT-14 reduces approximately 71.10, 93.12, 68.20 and 79.0% of Cr (VI), respectively, within 40 h incubation period (Table 3).

## Effect of Heavy Metals on Cr (VI) Reduction

Addition of different heavy metals at low concentration in the reduction medium did not affect significantly on the reduction potential of these strains. In all strains, 50 µg $ml^{-1}$ of $CoCl_2$ inhibited the Cr (VI) reduction proficiency of the strains to some extent while 200 µg $ml^{-1}$ $ZnSO_4$

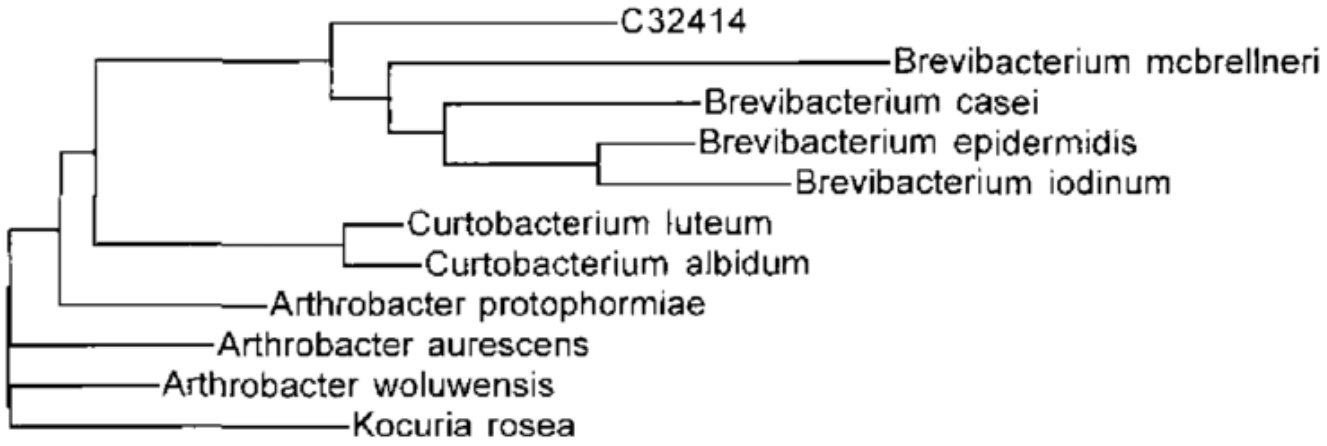



**Figure 5.** Phylogenetic relationship of *Bravibacterium* sp. CrT-13 (C32414) to other bacteria.

partially promoted the reduction of Cr (VI) in to Cr (III) by all strains. 200 µg $ml^{-1}$ $MnSO_4$ make some enhancement in the reduction ability of the strains (Table 3).

## DISCUSSION

All strains used in this study showed very high-level resistance against potassium chromate both in nutrient broth (up to 25 mg $ml^{-1}$) and on nutrient agar (40 mg $ml^{-1}$). Such a high level resistance has not been previously reported. Chromium resistant bacteria isolated from effluent of tanneries could resist up to 250 µg $ml^{-1}$ of Cr (VI) in the medium (Basu et al., 1997). Megharaj et al. (2003) also reported strains, which were isolated from polluted soil, could resist up to 100 µg $ml^{-1}$ of Cr (VI). Besides, chromium strains have a broad range of heavy metals (Mn, Ni, Zn, Pb, Cu and Co) and antibiotics (streptomycin, ampicillin, tetracycline, kanamycin, Chloramphenicol) resistances, which shows a positive sign for the application of these strains in the treatment of industrial effluents. Strains reported by Filali et al., (2000) also exhibited different heavy metals and antibiotics resistances.

It was found that the amount of chromium accumulated by the cells increased with increase in concentration of chromium from 100 to 1000 µg $ml^{-1}$ $K_2CrO_4$. Actually our strains shows very high level resistance to chromate and majority of them could resist up to 40 mg $ml^{-1}$ of $K_2CrO_4$ on the nutrients agar plates. When we increase the initial chromate concentration from 100 to 500 µg $ml^{-1}$ the chromate content in the cell become almost doubled and same situation was observed by increasing the initial chromate concentration from 500 to 1000 µg $ml^{-1}$. The rate of chromate accumulation was initially rapid but maximum quantity of chromate was achieved after 4 hours incubation period. Overall results show that the living cells exhibited better chromate uptake compared to heat killed and dried cells.

All strains were able to reduce Cr (VI) after different incubation periods. The reduction of Cr (VI) has also recently been reported by others investigators. Bacterial strains such as *Desulfomicrobium norvegicum* (Michel et al., 2003), *Bacillus* (Camargo et al., 2003), *Shewanella* (Guha et al., 2001; Viamajala et al., 2003; Middleton et al., 2003) *Desulfovibrio* (Chardin et al., 2002), *Escherichia coli* (Puzon et al., 2002), *Pseudomonas* (McLean and Beveridge, 2001), and *Alcaligenes* (Peitzsch et al., 1998) have been reported to successfully reduce Cr (VI) into Cr (III). Our results revealed that different heavy metals at low concentration did not significantly affect the reduction potential of these strains but they might be problematic at higher concentrations. McLean and Beveridge (2001) also observed that at low concentrations copper (40 mg $L^{-1}$) and arsenic (20 mg $L^{-1}$) had no significant effects on the reduction potential of Pseudomonads (CRB5), but at high concentration (120 mg $L^{-1}$) arsenic strongly inhibit the Cr (VI) reduction. Hg and Ag have proven to be strong inhibitors in chromate reduction by *Pseudomonas putida* (Ishibashi et al., 1990). The hexavalent chromium reduction studies described by these investigators, however, were performed under different conditions but at a low level concentration, whereas the present work was performed at high concentrations (200 µg $ml^{-1}$) of different heavy metals.

Hexavalent chromium reduction potential of strain *Bravibacterium* sp. CrT-13 was almost better in both effluent sample I and II as compared to others strains. This strain reduces almost 72.12% and 93.12% of Cr (VI) in sample I and II, respectively after 40 h incubation period. Hardoyo and Ohtake (1991) have also observed Cr (VI) reduction in industrial effluent collected from metal finishing plant. They found that in *Enterobacter cloaca* HO1, significant inhibition in Cr (VI) reduction was observed when the effluent contain 220 µg $ml^{-1}$ of Cr (VI), 25 µg $ml^{-1}$ of Cu, 26 µg $ml^{-1}$ of Mn and 0.02 µg $ml^{-1}$ of Zn. In another study Ganguli and Tripathi, 2002, also observed the reduction of Cr (VI) present in electroplating effluent with the help of *Pseudomonas aeruginosa* A2Chr in two-bioreactor system. All strains but especially *Bravibacterium* sp. CrT-13 showed amazing performance in this respect, which suggests that a hexavalent chromium reduction system of this nature can be economical to run.

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